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Iron Toxicity to Peritoneal Macrophage Due to Alteration of Mitochondria by NO

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The cytotoxic effect of iron was examined in peritoneal macrophage to determine contributing factors by iron injection to rat. Viability was reduced by 24% by the iron-overload and by 30% by short-term iron addition. Total iron was increased by 45% in the iron-overloaded with remarkable elevation (9 to 14 fold) in the presence of $FeSO_4$. Free calcium was also increased by 19% in control and 44% in iron-overloaded group due to additional $FeSO_4$. NO and MDA were increased by 40% and 136%, respectively, with significant reduction (37%) of NAD(P)H. RCR and cytochrome c oxidase activity were lowered approximately by 10% with reduction of mitochondrial membrane potential. Addition of iron was frequently associated with altered distribution of mitochondria of high membrane potential in the iron-overloaded macrophage. These results suggest altered mitochondria with high NO and low NAD(P)H due to iron.

Iron, as an active transition metal ion, is necessary for many biological processes. Iron plays an essential role in maintenance of mitochondria, through its functional forms, heme and iron-sulfur clusters. Heme and iron-sulfur clusters are important in normal assembly and activity of electron transport system. Impairment of mitochondrial function can result from abnormal homeostasis of iron (Atamna et al., 2002; Pandolfo, 2002). It is potentially detrimental to the cell as it generates reactive oxygen species by Fenton reaction. Iron effect is especially profound in lipid peroxidation that alters membrane structure (Gutteridge, 1982). Iron excess may contribute to major pathological processes such as cancer, atherosclerosis, rheumatoid arthritis, and neurodegenerative diseases (Halliwell and Gutteridge, 1990). However, many aspects of which iron influences these processes at the molecular and cellular level are unclear.

Iron and iron binding proteins have immunoregulatory properties with possibly severe physiological effects by iron excess and deficiency (Walker and Walker, 2000). Differential responses may be mediated by modulation of iron-dependent metabolic functions according to distribution of iron in various types of immune cells (Kovjazin et al., 2003). Macrophage actively clears excessive iron by Nramp1 (Natural resistance associated macrophage protein 1) (Mulero et al., 2002), which would be beneficial for the organism by limiting the iron supply to infective microorganism and by reducing generation of harmful reactive oxygen species (Alford et al., 1991). Conversely, iron accumulation can damage the macrophage itself. Macrophage, active in iron transport, produces NO by inducible NO synthase (iNOS) with modulation of immune function (Ding et al., 1988; Macmicking et al., 1997; Brock and Mulero, 2000). Through its interaction with components of the electron transport chain, NO might function not only as a physiological regulator of cell respiration, but also as an enhancer of production of reactive oxygen species such as peroxynitrite (Brown, 2001). In fact, NO effect can be better understood through its interaction with mitochondria.

In this work, we tried to determine the primary factors responsible for cytotoxicity of iron in macrophages in relation to mitochondrial functions.

Materials and Methods

Iron injection, preparation and culture of peritoneal macrophage, and NO assay

Sprague-Dawley rats (8 weeks old, 180-200 g), purchased from Daehan Biolink (Umsung), were injected hypodermically with $FeCl_3$ (0.049 mg/g body weight) three times every other day. Control rats were injected with an equal amount of saline. During the last injection, thioglycollate broth (Difco) (0.1 ml/g body weight) was

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injected intraperitoneally. Three days later, the rats were anesthetized with ether. For preparation of peritoneal macrophage, 25 ml of ice-cold RPMI 1640 (Sigma) was added into peritoneum and macrophages were obtained after centrifugation at 600×g for 10 min using Histopaque (Sigma). To study the iron effect in cultured cells, peritoneal macrophages were prepared from control and iron-injected rats (which we call 'iron-overload') as above. After washing with ice-cold PBS solution, the cells were incubated at 37°C, 5% CO_2 for 1 h for attachment with fresh media of RPMI 1640 containing 10% FBS and 100 μg/ml penicillin-streptomycin (Biowhittaker) as in Manual of Macrophage Methodology (Marcel Dekker, 1981). For iron effect, after cell attachment, 200 μM $FeSO_4$ was added to the control and iron-overload cultures. After 2 h, media containing $FeSO_4$ were replaced with fresh medium without $FeSO_4$. Every 24 h, aliquots were stained with Trypan blue and viable cells were counted. NO in the media was measured using Griess reagents (Green et al., 1982) after 1 h incubation following replacement with fresh media on the first day of primary culture.

Measurement of iron and calcium content

For macrophages prepared from the control and iron-overload groups, total iron was measured at 520 nm after incubation of sonicated homogenates at 95°C, for 30 minutes in 75 mM Na_2SO_3, 0.05% bipyridyl, 6% (v/v) acetic acid (Levi et al., 1989). For calcium measurement, 40 μM arsenazo III was added to macrophages in PBS after sonication for 3 min and the absorption difference at 665 nm and 685 nm was obtained (Gorman et al., 1978).

Measurement of lipid peroxidation (MDA), and NAD(P)H

To macrophages, 20% trichloroacetic acid and 1% 2-thiobarbituric acid were added and incubated at 95°C for 30 min and absorbance was measured at 532 nm to detect malondialdehyde (MDA) generated from lipid peroxidation (Draper and Hadley, 1990). NAD(P)H was measured with cell homogenates prepared by sonication in PBS using spectrofluorometer (Shimadzu, Japan) for excitation at 340 nm and emission at 465 nm (Masini et al., 1987). For all the measurements, the number of cells was adjusted to 1×10^6.

Respiratory control ratio (RCR), cytochrome c oxidase (CcO) activity, and membrane potential of mitochondria

Mitochondria were prepared by differential centrifugation from macrophages after incubation in preparation buffer (0.25 M sucrose, 3 mM Hepes, 1 mM EDTA, and 0.05% saponin, pH 7.4) for 10 min on ice. RCR was measured using Clark type oxygen electrode at 25°C in 0.25 M sucrose, 10 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, 20 mM KCl, 20 mM Tris-Cl (pH 7.4), and 1 mM EDTA using 1 mg of mitochondria (Eastabrook, 1967). Succinate and ADP were added during measurement to final concentrations of 12 mM and 0.3 mM, respectively. Cytochrome c oxidase activity was assayed after lysing macrophages (1×10^6 cells) in 0.05% saponin in PBS at room temperature for 20 min. With reduced cytochrome c (horse type VI) (10 μM) in 0.1 M sodium phosphate (pH 7.0), in the presence of 10 μM ascorbate, 200 ng/ml catalase and 400 μM 3,3'diaminobenzidine-tetrachloride (DAB) were added and the absorbance was measured at 450 nm for 15 min in the absence or presence of 100 μM potassium cyanide (Chrzanowska-Lightowlers et al., 1993). Mitochondrial membrane potential was measured in macrophage using rhodamine 123 (Johnson et al., 1980). Macrophages (1×10^6 cells) in 200 μl PBS buffer were mixed with 5 μg/ml of rhodamine 123. For fluorescence microscopy (Leica), cells were incubated for 10 min at 37°C in the CO_2 incubator after the addition of rhodamine 123.

Immunoblot assay

Twenty microgram of sonicated macrophages were used for slot blot on nitrocellulose membrane and antibody for iNOS (Santacruz) were added. Fast 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) was added for the immunodetection.

All the chemicals were purchased from Sigma, except for cell media (Difco).

Results

Cell viability and iron and calcium content

Peritoneal macrophages were prepared from either control or iron-overload rats. Aliquots of primary cultures on 6-well plate were incubated for 2 h in the absence or presence of 200 μM $FeSO_4$, and after 1 day, the cells were counted. As expected, the number of macrophages was increased by 26% by iron (3.38×10^6 in control vs 4.22×10^6 in iron-overload in Table 1). However, the viability of macrophage from iron-overload group declined significantly after 1 day compared with that of control rat (63% was viable in control vs 39% in iron-overload). However, the direct effect of iron addition to primary culture

Table 1. Cell viability of macrophages from control and iron-overloaded rats*

Days	Control		Iron-overload	
	-$FeSO_4$ (%)	+ $FeSO_4$ (%)	-$FeSO_4$ (%)	+ $FeSO_4$ (%)
1	3.38(100)	3.38(100)	4.22(100)	4.22(100)
2	2.13(63±5)	1.13(33±16)	1.65(39±8)	1.98(47±9)

*Cell numbers are shown as 10^6 cells/ml. The data are means±S.D. of 5 independent experiments.

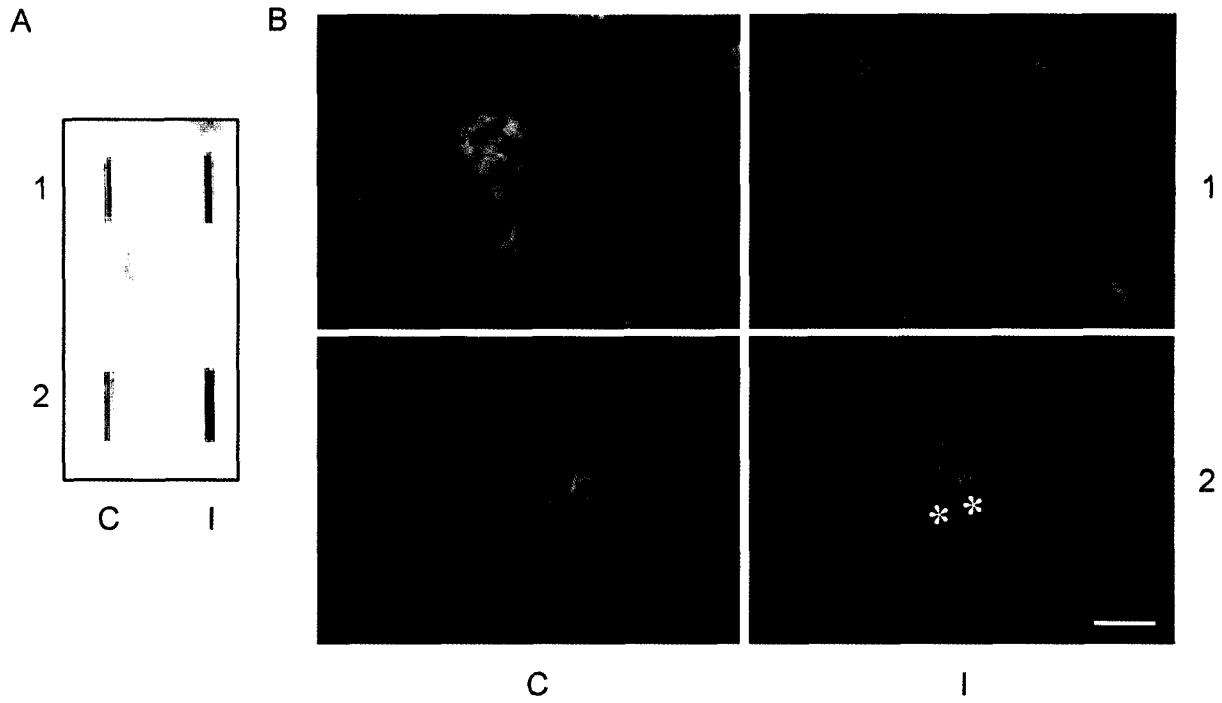

Fig. 1. Immunodetection of iNOS and membrane potential of macrophage. A, Immunodetection of iNOS from cell lysates of primary cultures of macrophage. Cell homogenates were prepared from control rat macrophages in the absence (C1) or presence of $FeSO_4$ (C2), and from iron-overloaded rat cells in the absence (I1) or presence of $FeSO_4$ (I2). B, Fluorescence micrograph of peritoneal macrophages stained by rhodamine 123 in the absence or presence of iron. Reddish yellow color corresponds to high membrane potential by rhodamine 123 uptake whil e greenish yellow to low potential. C1, control; C2, control in the presence $FeSO_4$; I1, iron-overload; I2, iron-overload in the presence of $FeSO_4$. In iron-overload cells in the presence of $FeSO_4$ (I2), grain-like structures indicated by star were observed. Scale bar=50 μm.

was different in the two groups; control cells were susceptible to added iron as viable cells were decreased to almost half (33% vs 63%, Table 1) whereas the iron-overload cells did not show much difference (47% vs 39%). Though the cause of this opposite effect is not known, NO in response to $FeSO_4$ addition seems to be responsible. NO amount was even less in the presence of $FeSO_4$ in the control macrophage, whereas it was increased to a great extent by $FeSO_4$ in the iron-overload cells (Table 3), suggesting activation of iNOS. This result can be explained by NO-mediated protection (Chung et al., 2001). But the viability was quickly dropped in the iron-overloaded after longer incubation (data not shown). As macrophages are actively proliferating with a short half-life, the damaged cells can be easily replaced *in vivo*. But this system may not persist *in vitro*.

Total iron was measured in primary cultures of macrophages after medium washing (Table 2). The augmentation was significant in the iron-overloaded (10.32 μM) compared with control (7.14 μM). Particularly, the short-term addition of $FeSO_4$ showed a dramatic increase (more than 9 fold) both in the control and iron-overloaded macrophage, consistent with the great capacity for iron clearance *in vivo* (control 7.14 μM vs 102.57 μM, and iron-overload 10.32 μM vs 93.98 μM). The calcium content by $FeSO_4$ addition *in vitro* was similarly changed as the iron content, though to a much less extent. When the iron content was compared in spleen, it was

Table 2. Iron and calcium content of macrophage after iron addition

Group	Fe (mM)*	Ca (nM)*	Fe:spleen (mM)**
Control	7.14±2.44	44.90±11.03	3.9±1.5
Control+$FeSO_4$ (*in vitro*)	102.57±21.78	53.30±19.37	
Iron-overload	10.32±4.67	40.20±10.32	6.1±2.6
Iron-overload+$FeSO_4$ (*in vitro*)	93.98±25.78	57.70±3.57	

*The data are means±S.D. of 6 independent experiments and standardized for 1×10^6 cells per ml. $P<0.05$ by Student's t-test. **The data are means of S.D. of 3 independent experiments with units of mM per g of tissue.

Table 3. NO, NAD(P)H and MDA (lipid peroxidation) in macrophage after iron addition

Group	NO*	NAD(P)H**	MDA*
Control	4.05±2.07	19.47±6.53	0.61±0.03
Control+$FeSO_4$ (*in vitro*)	3.13±2.07	15.87±4.45	3.84±1.24
Iron-overload	5.69±3.55	12.18±5.02	1.44±0.03
Iron-overload+$FeSO_4$ (*in vitro*)	7.09±5.00	12.71±4.72	3.05±0.70

The data are means±S.D. of 6 independent experiments and standardized for 1×10^6 cells. *(nmole/mg protein) **fluorescence emission at 465 nm.

increased by 60% in the iron-overloaded rat demonstrating active transport/deposit by macrophage (Table 2).

Measurement of NO, lipid peroxidation, and NAD(P)H

NO was increased by 40% in iron-overload rats (4.05 vs 5.69 nmole) (Table 3). However, short-term addition of $FeSO_4$ *in vitro* gave opposite effect, i.e., decrease in control (4.05 vs 3.13 nmole) but increase in iron-overload (5.69 vs 7.09 nmole). Though the responsible factors for the variation are not presently known, isoforms of NO synthase (NOS) may be differentially activated in macrophage depending on the condition of iron addition. Lipid peroxidation was compared in cell homogenates from primary cultures in the absence or presence of iron (Table 3). Addition of $FeSO_4$ enhanced MDA more than 6-fold in control (3.84 vs 0.61 nmole) and 2-fold in iron-overload (3.05 vs 1.44 nmole), with little change of NAD(P)H (15.87 vs 19.47 in control and 12.71 vs 12.18 in iron-overload). When iNOS was compared with cell homogenates from primary cultures using antibody against iNOS, the amount of iNOS was increased in an iron-overload group (Fig. 1A) in agreement with high NO content in Table 3. Additional iron enhanced the iNOS slightly suggesting posttranscriptional activation consistent with NO increase.

Respiratory control ratio (RCR), cytochrome c oxidase (CcO) activity and membrane potential

RCR is considered as an index of functional integrity of mitochondria. If membrane structure is damaged, RCR would be lower. As lipid is an essential component of membrane function and structure, iron can affect the membrane properties through lipid peroxidation. RCR and CcO activity were decreased by approximately 10% in the iron-overloaded group (Table 4). The difference seems slight probably due to the assay on the whole cell, not isolated mitochondria. With isolated mitochondria from other tissues, the decrease was considerable (unpublished data).

Mitochondria are the main energy supplier in a cell with membrane potential. As the uptake of fluorescent rhodamine 123 is proportional to the membrane potential, mitochondrial energy state can be compared by the fluorescent intensity. The overall intensity in fluorescence micrograph from green to yellow was low in the iron-overloaded cell (Fig. 1B). However, intense fluorescence (yellow) along with clustered form was frequently observed in the iron-overload macrophages in the presence of iron. This may reveal mitochondria with increased membrane potential associated with cytochrome c release (Hortelano et al., 1999). When this occurred, the mitochondria were not uniformly distributed throughout the cytoplasm but, more or less, centered and clustered to each other with disappearance of sharpness of cell boundary and vesicles characteristic to macrophage.

Table 4. RCR and CcO activity in macrophage

Group	RCR	CcO activity*
Control	1.29±0.15	0.077±0.004**
Iron-overload	1.13±0.05	0.069±0.006**

The data are means±S.D. of 4 independent experiments.
*CcO activity is expressed in absorbance at 450 nm per mg protein.
**$P<0.01$ by Student's t-test.

Discussion

Macrophage serves as a guard protecting the organism from harsh environment. It is particularly active in transporting labile iron to its final depot such as spleen (Weiss, 2002). As excessive iron produces harmful oxygen radicals (McCord, 1998), under iron-overload, cells would suffer from free radicals. As an immune cell, cell death would liberate the signal to stimulate proliferation for better protection. The 30% increase in cell number in the iron-overloaded compared with control (Table 1) seems to be minimum as attachment of cells from the iron-overload was less efficient (unpublished observation). The actively proliferating macrophages would replace the damaged cells, as shown by the abrupt drop of cells in iron-overload and control in the presence of $FeSO_4$ (Table 1).

A great capacity of rapid uptake of labile iron by macrophage was revealed by the iron accumulation during 2-h incubation with $FeSO_4$ in both iron-overloaded and control macrophages (Table 2). Low molecular mass iron pool supplies iron for the biosynthesis of essential proteins. For example, mitochondria have been reported to take up iron rapidly and may have small 'pools' of non-protein-bound iron ions in the matrix (Schueck et al., 2001; Pandolfo, 2002). Studies using fluorescent iron chelators support this. However, the size and chemical nature of this 'non-protein-bound iron pool' in cells and organelles is unknown. Iron in macrophage is transported by DCT 1 (Divalent cation transporter 1), formerly Nramp2 (Weiss, 2002), and stored in vesicle, which should be released into spleen for systemic utilization. Probably, the iron content in cytoplasmic ferritin and mitochondria would be relatively low compared with other types of cells such as hepatocyte. In particular, immediate uptake of iron by isolated macrophages was great compared to the increase of iron content when iron was injected over a week (Table 2). This result can be explained by the fact that of iron macrophages are active in transport and not in storage (Brissot et al., 2002). Apparently, some of the ingested iron would stimulate proliferation for large population of macrophage. Thus damaged cell may be removed and replaced by new one with low iron content, as shown in low iron content in long-term iron injection (iron overload) compared with that in short-term addition (control with

$FeSO_4$).

Iron can influence the membrane structure through lipid peroxidation (Romslo and Flatmark, 1973). The extent of lipid peroxidation was changed in parallel with iron content, which was particularly high in the iron-overloaded rat compared with control (Table 2). Similar correlation was found in testis (Lucesoli and Fraga, 1995). Lipid peroxidation probably, accumulates in macrophage, while iron content may fluctuate during delivery. Cardiolipin is a unique kind of lipid located in the inner membrane of mitochondria. If cardiolipin is oxidized, cytochrome c can be liberated to activate apoptotic pathway (Shidoji et al., 1999). Also, as a structural and functional component of the inner membrane, peroxidation of cardiolipin can be detrimental to electron transport system including cytochrome c oxidase and ATP translocator. If the antioxidants and ATP are exhausted, the damage cannot be repaired in time. This kind of catastrophe linked to death pathway will lead to necrosis or apoptosis. Membrane-associated function might as well be altered to impair the oxidative phosphorylation and to decrease the membrane potential due to iron or lipid peroxidation (Hanstein et al., 1981; Masini et al., 1994). Decreased RCR could be ensued by fatty acid liberated from membrane breakdown (Table 4). Damaged membrane would augment reactive oxygen species from electron transport system. This oxidative stress may also affect the pore opening via mitochondria permeability transition (MPT) and diminished membrane potential (Gogvadze et al., 2003). Reduced energized state of mitochondria, as shown in Fig. 1B, seems to be related with the change in localization of mitochondria, from membrane boundaries to near center in the iron-overloaded macrophage. Aggregated form, seemingly the altered (giant) structure of mitochondria, was often found in the presence of additional iron in iron-overload macrophage (Fig. 1B). Similar alteration was observed in rat hepatocyte with excess iron (Romslo and Flatmark, 1973). Presently the physiological meaning of this phenomenon is not understood. It may be a salvage process that reconstructs large mitochondria by combining feeble areas around the nucleus as in old cell or in pathological state (Hagen et al., 1997).

To be repaired from the damage by lipid peroxidation, reducing power would be necessary. NAD(P)H is a major reducing factor, which was decreased considerably in the iron-overloaded macrophage (Table 3). As it is essential to maintain a critical level of NAD(P)H, alternate way may be operated in mitochondria in the iron-overloaded macrophage such as reverse electron transport by sacrificing ATP via reverse of ATP synthase (Romslo and Flatmark, 1975). In addition, uptake of divalent ion including iron by DCT1 is coupled to proton (Gunshin et al., 1997). Iron uptake could be mediated by Fe-ATPase with ATP loss (Baraño et al., 2000). Taken together, iron overload would result in declined membrane potential as in Fig. 1B. Calcium content was changed similarly with iron content, suggesting a metabolic link between iron and calcium (Seligman et al., 1991). Alternatively, activation of guanyl cyclase by NO can stimulate calcium release (Kolb et al., 1994).

NO is a free radical with a strong affinity for iron. NO modulates iron-dependent enzymes, including cis-aconitase (Drapier et al., 1993). Targeted at the iron-sulfur center, the enzyme becomes IRP1 (Iron regulatory protein1) and regulates both ferritin mRNA translation and transferrin mRNA stability, thus directly affecting iron metabolism. Further investigations revealed an inseparable relationship between iron and NO in an autoregulatory cycle (Weiss et al., 1995). Indeed, nonheme labile iron induces NO production (Kim et al., 2000). Physiological significance of the NO might be to eliminate harmful labile iron through binding (Kim and Ponka, 2002). Indeed iNOS activity with concomitant high NO content is enhanced in the presence of iron in the iron-overloaded macrophage (Fig. 1A; Table 3). If NO was not produced in time, then NO content would decrease momentarily due to iron binding (Zhang et al., 1998). This may occur in control rat in the presence of iron (Table 3).

NO synthesis will accompany NAD(P)H consumption by NO synthase (NOS), as in iron-overloaded rat (Table 3). However, NAD(P)H level was not changed by additional iron treatment suggesting it already reached basal level. Maintenance of reducing power would require reverse electron transport by ATPase activity of ATP synthase. This ATP loss can cause cell death as revealed in low membrane potential (Fig. 1B). Hence the price of NO production could be the inevitable loss of NAD(P)H, which was accelerated by the aforementioned lipid peroxidation. Similar reduction of ATP level due to glutathione-NO complex (GSNO) was observed during uptake of iron from transferrin (Watts and Richardson, 2000).

In mitochondria, NO acts as an oxygen sensor for cytochrome c oxidase (Brown, 2001) and cytochrome c release (Brookes et al., 2000). Accordingly, NO regulates overall electron transport system and energy production of mitochondria. Mitochondria permeability transition (MPT) is similarly inhibited (Brookes et al., 1999). These effects suggest that NO freezes mitochondrial function, which should be beneficial in protecting the vulnerable cell from mitochondria-mediated death pathway (Di Lisa et al., 1998). NO gas generated by iNOS in macrophage can penetrate mitochondrial membrane. In this context the recently identified mtNOS (Ghafourifar and Richter, 1997; Tatoyan and Giulivi, 1998) would be appropriate to elicit immediate response inside the mitochondria (Lopez-Figueroa et al., 2000), but it needs further investigation. By forming nitrosyl-iron complex, substrate binding can be prevented leading to inhibition of Complex I and II. However, above the physiological concentration, the protective role of NO may not sustain but NO may promote cell death via formation of harmful peroxynitrite.

In fact, NO increased membrane potential after cytochrome c release (Hortelano et al., 1999) demonstrating a dual role of NO in diverse pathways leading to either cell death or cell survival (Chung et al., 2001).

Taken together, the present result suggests iron uptake in macrophage consumes NAD(P)H by directly regulating NO synthesis by iNOS, lipid peroxidation, and calcium cycling. NO, in turn, exacerbates the situation by increasing membrane potential after cytochrome c release from mitochondria (Moncada and Erusalimsky, 2002). High membrane potential would accelerate the generation of free radicals (Liu, 1999). To keep the basal level of NAD(P)H, reverse electron transport would occur accompanied by ATP consumption. Thus, through low NAD(P)H and low ATP associated with altered membrane potential, NO production from iron uptake would cause devastating effects primarily on mitochondria and lead to cell death, supporting the idea that mitochondria are the major responding organelle determining cell survival and death (Hortelano et al., 1999).

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References

Alford CE, King TE, and Campbell PA (1991) Role of transferrin, transferrin receptors, and iron in macrophage listericidal activity. *J Exp Med* 175: 459-466.

Anderson PG (2000) Concentration-dependent effects of nitric oxide on mitochondria permeability transition and cytochrome c release. *J Biol Chem* 275: 20474-20479.

Atamna H, Walter PB, and Ames BN (2002) The role of heme and iron-sulfur clusters in mitochondrial biogenesis, maintenance, and decay with age. *Arch Biochem Biophys* 397: 345-353.

Barañano DE, Wolosker H, Bae B-I, Barrow RK, Snyder SH, and Ferris CD (2000) A mammalian iron ATPase induced by iron. *J Biol Chem* 275: 15166-15173.

Brissot P, Pigeon C, and Loreal O (2002) Regulation of systemic iron transport and storage. In: Templetion DM(ed) Molecular and Cellular Iron Transport, Marcel Dekker, Inc., Basel.

Brock JH and Mulero V (2000) Cellular and molecular aspects of iron and immune function. *Proc Nutr Soc* 59: 537-540.

Brookes PS, Bolanos JP, and Heales SJ (1999) The assumption that nitric oxide inhibits mitochondrial ATP synthesis is correct. *FEBS Lett* 446: 261-263.

Brookes PS, Salinas EP, Darley-Usmar K, Eiserich JP, Freeman BA, Darley-Usmar VM, and Brown GC (2001) Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. *Biochim Biophys Acta* 1504: 46-57.

Chrzanowska-Lightowlers ZM, Turnbull DM, and Lightowlers RN (1993) A microtiter plate assay for cytochrome c oxidase in permeabilized whole cells. *Ann Biochem* 214: 45-49.

Chung H-T, Pae H-O, Choi B-M, Billiar TR, and Kim Y-M (2001) Nitric oxide as a bioregulator of apoptosis. *Biochem Biophys Res Comm* 282: 1075-1079.

Di Lisa F, Menabo R, Canton M, and Petronilli V (1998) The role of mitochondria in the salvage and the injury of the ischemic myocardium. *Biochim Biophys Acta* 1366: 69-78.

Ding AH, Nathan CF, and Stuehr DJ (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. *J Immunol* 141: 2407-2412.

Draper HH and Hadley M (1990) Malondialdehyde determination as index of lipid peroxidation. *Meth Enzimol* 186: 421-431.

Drapier JC, Hirling H, Wietzerbin J, Kaldy P, and Kuhn LC (1993) Biosynthesis of nitric oxide activates iron regulatory factor in macrophages. *EMBO J* 12: 3643-3649.

Eastabrook RW (1967) Mitochondrial respiratory control and the polarographic measurement of ADP : O ratios. *Meth Enzimol* 10: 41-47.

Ghafourifar P and Richter C (1997) Nitric oxide synthase activity in mitochondria. *FEBS Lett* 418: 291-296.

Gogvadze V, Walter PB, and Ames BN (2003) The role of Fe2+-induced lipid peroxidation in the initiation of the mt permeability transition. *Arch Biochem Biophys* 414: 255-260.

Gorman AC and Thomas MV (1978) Changes in the intracellular concentration of free calcium ions in a pace-maker neurine, measured with the metallochromic indicator dye arsenazo III. *J Physiol* 275: 357-376.

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS and Tannenbaum SR (1982) Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Ann Biochem.*126: 131-138.

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron VF, Mussberger S, Gollan JL, and Hediger MA (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* 388: 482-488.

Gutteridge JMC (1982) The role of O2'- and OH' in phospholipid peroxidation catalyzed by Fe salts. *FEBS Lett* 150: 454-458.

Halliwell B and Gutteridge JMC (1990) Role of free radicals and catalytic metal ions in human disease: an overview. *Meth Enzimol* 186: 1-85.

Hagen TM, Yowe DL, Bartholomew JC, Wehr CM, Do KL, Park JY, and Ames BN (1997) Mitochondrial decay in hepatocytes from old rats: membrane potential declines, heterogeneity and oxidants increase. *Proc Natl Acad Sci USA* 94: 3064-3069.

Hanstein WG, Heitmann TD, Sandy A, Biesterfeldt HL, Liem HH and Muller-Eberhard U (1981) Effects of hexachlorobenzene and iron loading on rat liver mitochondria. *Biochim Biophys Acta* 678: 293-299.

Hortelano S, Alvarez AM, and Bosca L (1999) Nitric oxide induces tyrosine nitration and release of cytochrome c preceding an increase of mitochondria transmembrane potential in macrophages. *FASEB J* 13: 2311-2317.

Johnson LV, Walsh ML, and Chen LB (1980) Localization of mitochondria in living cells with rhodamine 123. *Proc Natl Acad Sci USA* 77: 990-994.

Kim Y-M, Chung H-T, Simmons RL, and Billiar TR (2000) Cellular non-heme iron content is a determinant of nitric oxide-mediated apoptosis, necrosis, and caspase inhibition. *J Biol Chem* 275: 10954-10961.

Kim SW and Ponka P (2002) Nitrogen monoxide-mediated control of ferritin synthesis: Implications for macrophage iron homeostasis. *Proc Natl Acad Sci USA* 99: 12214-12219.

Kolb JP, Paul-Eugene N, Damais C, Yamaoka K, Drapier JC, and Dugas B (1994) Interleukin-4 stimulates cGMP production by IFN-gamma-activated human monocytes. Involvement of the nitric oxide synthase pathway. *J Biol Chem* 269: 9811-9816.

Kovjazin R, Eldar T, Patya M, Vanichkin A, Lander HM, and Novogrodsky A (2003) Ferrocene-induced lymphocyte activation

and anti-tumor activity is mediated by redox-sensitive signaling. *FASEB J* 17: 467-469.

Levi S, Salfeid J, Francesca F, Cozzi A, Dorner MH, and Arosio P (1989) Expression and structural and functional properties of human ferritin L-chain from *E. coli*. *Biochemistry* 28: 5179-5184.

Liu S-S (1999) Cooperation of a "Reactive Oxygen Cycle" with the Q cycle and the proton cycle in the respiratory chain-superoxide generating and cycling mechanisms in mitochondria. *J Bioener Biomemb* 31: 367-376.

Lopez-Figueroa MO, Caamano C, Morano MI, Ronn LC, Akil H, and Watson SJ (2000) Direct evidence of NO presence within mitochondria. *Biochem Biophys Res Comm* 272: 129-133.

Lucesoli F and Fraga CG (1995) Oxidative damage to lipids and DNA concurrent with decrease of antioxidants in rat testes after acute iron intoxication. *Arch Biochem Biophys* 316: 567-571.

Macmicking J, Xie QW, and Nathan C (1997) NO and macrophage function. *Ann Rev Immunol* 15: 323-350.

Masini A, Trenti T, Ceccarelli D, and Muscatello U (1987) The effect of a ferric iron complex on isolated rat-liver mitochondria III. Mechanistic aspects of iron-induced calcium efflux. *Biochim Biophys Acta* 891: 150-156.

Masini A, Ceccarelli D, Gallesi D, Giovannini F, and Trenti T (1994) Lipid hydroperoxide induced mitochondrial dysfunction following acute ethanol intoxication in rats. The critical role for mitochondrial reduced glutathione. *Biochem Pharmacol* 47: 217-224.

McCord J (1998) Iron, free radicals and oxidative injury. *Semin Hematol* 35: 5-12.

Moncada S and Erusalimsky JD (2002) Does nitric oxide modulate mitochondria energy generation and apoptosis? *Nature Rev Mol Cell Biology* 3: 214-220.

Mulero V, Searle S, Blackwell JM, and Brock JH (2002) Solute carrier 11a1 (Slc11a1; formerly Nramp1) regulates metabolism and release of iron acquired by phagocytic, but not transferrin-receptor-mediated, iron uptake. *Biochem J* 363: 89-94.

Pandolfo M (2002) Iron metabolism and mitochondria abnormalities in Friedreich ataxia. *Blood Cells Mol Dis* 29: 536-547.

Romslo I and Flatmark T (1973) Energy-dependent accumulation of iron by isolated rat liver mitochondria. I. General features. *Biochim Biophys Acta* 305: 29-40.

Romslo I and Flatmark T (1975) Energy-dependent accumulation of iron by isolated rat liver mt. 5. Effect on factors controlling respiration and oxidative phosphorylation. *Biochim Biophys Acta* 387: 80-94.

Schueck ND, Woontner M, and Koeller DM (2001) The role of the mitochondrion in cellular iron homeostasis. *Mitochondrion* 1: 51-60.

Seligman PA, Kovar J, Schleicher RB, and Gelfand EW (1991) Transferin-independent iron uptake supports B lymphocyte growth. *Blood* 78: 1526-1531.

Shidoji Y, Hayashi K, Komura S, Ohishi N, and Yagi K (1999) Loss of molecular interaction between cytochrome c and cardiolipin due to lipid peroxidation. *Biochem Biophys Res Comm* 264: 343-347.

Tatoyan A and Giulivi C (1998) Purification and characterization of a nitric-oxide synthase from rat liver mitochondria. *J Biol Chem* 273: 11044-11048.

Walker EM Jr. and Walker SM (2000) Effects of iron overload on the immune system. *Ann Clin Lab Sci* 30: 354-365.

Walter PB, Knutson MD, Paler-Martinez A, Lee S, Xu Y, Viteri FE, and Ames BN (2002) Iron deficiency and iron excess damage mitochondria and mtDNA in rats. *Proc Natl Acad Sci USA* 99: 2264-2269.

Watts RN and Richardson DR (2000) Examination of the mechanism of action of nitrogen monoxide on iron uptake from transferrin. *J Lab Clin Med* 136: 149-156.

Weiss G, Wachter H, and Fuchs D (1995) Linkage of cell-mediated immunity to iron metabolism. *Immunol Today* 16: 495-500.

Weiss G (2002) Chap. 19. Iron acquisition by the reticuloendothelial system. In: Templeton DM (ed), Molecular and Cellular Iron Transport, Marcel Dekker, Inc, Basel, pp 467-486.

Zhang Y, Crichton RR, Boelaert JR, Jorens PC, Herman AG, Ward RJ, Lallemand F, and de Witte P (1998) Decreased release of nitric oxide (NO) by alveolar macrophages after *in vivo* loading of rats with either iron or ethanol. *Biochem Pharmacol* 55: 21-25.

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